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Citation: McGuire, Karen, Jamison, James, Gilloteaux, Jacques and Summers, Jack (2013) Vitamin C and K3 Combination Causes Enhanced Anticancer Activity against RT-4 Bladder Cancer Cells. *Journal of Cancer Science & Therapy*, 05 (10). pp. 325-333. ISSN 1948-5956

Published by: Omics Publishing Group

URL: <http://dx.doi.org/10.4172/1948-5956.1000223> <<http://dx.doi.org/10.4172/1948-5956.1000223>>

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# Vitamin C and K<sub>3</sub> Combination Causes Enhanced Anticancer Activity against RT-4 Bladder Cancer Cells

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## Abstract

**Introduction:** Vitamin C (VC), Vitamin K<sub>3</sub> (VK<sub>3</sub>) and the combination (VC:VK<sub>3</sub>) were evaluated against human bladder cancer cell lines RT-4 and T24 to evaluate their synergistic anticancer activity.

**Methods/Results:** An MTT assay compared a 1hr pulsed versus a 5hr continuous exposure. VC:VK<sub>3</sub> was synergistic, increasing the antitumor activity 12- to 24 fold for RT-4 cells. VC:VK<sub>3</sub> pulsed versus continuous exposure produced comparable CD<sub>50</sub> values, indicating a triggered response involving a catalase reversible redox mechanism generating hydrogen peroxide. Hydrogen peroxide production caused lipid peroxidation and depletion of cellular thiols. ATP levels were measured over 5hrs to determine metabolic effects where VC:VK<sub>3</sub> caused a unique spike in ATP levels. Though the cause of the ATP spike is unknown a possible mechanism is a shunt formed around a defective region of complex III of the ETC from coenzyme Q to cytochrome c, producing a shift from glycolytic to oxidative metabolism and a diminution of lactic acidosis. Analysis of mitochondrial and extra mitochondrial calcium levels revealed a unique calcium pattern for RT4 cells treated with CD<sub>50</sub> doses of VC, VK<sub>3</sub> or VC:VK<sub>3</sub>.

**Conclusion:** VC:VK<sub>3</sub> was able to cause autoshizic cell death through oxidative stress, thiol depletion, lipid peroxidation, modification of ATP levels and calcium regulation. Because of these results, VC:VK<sub>3</sub> was granted orphan drug status for the treatment of metastatic or locally advanced, inoperable transitional cell carcinoma of the urothelium (stage III and IV bladder cancer). Efforts are underway to conduct a phase II clinical trial for this indication.

**Keywords:** Cancer cell lines; Chemokine; H<sub>2</sub>O<sub>2</sub>; Vitamin C (VC); Vitamin K<sub>3</sub> (VK<sub>3</sub>)

## Introduction

The latest statistics from the National Cancer Institute estimates that 72,570 new cases of bladder cancer will be diagnosed in the United States in 2013 and will result in 15,210 deaths. Bladder cancer is six times more prevalent in developed countries than in under developed countries and is the fifth most common human malignancy. Bladder cancer is also one of the most expensive cancers to treat since the course of therapy requires extensive patient surveillance to monitor for recurrence as well as repeated procedures to remove new tumors or cryptic tumor foci overlooked during the initial transurethral resection [1-4]. These urothelial carcinomas are primarily of epithelial origin (>90%) with multiple genetic pathways leading to disease progression [5]. Patients with high-risk non-muscle invasive bladder cancer receive adjuvant Bacillus Calmette-Guérin (BCG) therapy alone or in combination with interferon α-2b [6], radiation and/or chemotherapy, typically methotrexate, vinblastine, doxorubicin and cisplatin (MVAC), or other targeted strategies [7]. Even with the latest pharmacologic strategies, the relative survival rate for bladder cancer is 5 years, while the median survival for patients with inoperable metastatic bladder cancer is 7 to 20 months [5,8,9].

A new paradigm in cancer therapy is slowly gaining popularity and continues to evolve from the work of Roger Daoust and Henryk Taper [10-13]. Daoust studied the DNase I and DNase II expression patterns of a variety of tumor types and discovered that DNases were often suppressed in tumor cells, despite being active in the surrounding tissues and vasculature. Daoust also found that reactivation of both DNases was associated with successful cancer treatment or spontaneous cancer remission. This work was extended by Henryk Taper and co-workers [14,15] for the treatment of liver and other cancers by using

a combination of vitamin C and vitamin K<sub>3</sub> (VC:VK<sub>3</sub>) in a 100:1 ratio. Taper showed that vitamin C reactivated DNase II, while vitamin K<sub>3</sub> reactivated DNase I with the combination synergistically causing tumor cell death. Further experimentation showed that VC:VK<sub>3</sub> was an effective chemo- and radio-sensitizer [11-13]. Subsequently, these studies were extended to include bladder and [16-22] other cancers [23-27].

Unlike the majority of chemotherapeutic agents which target rapidly dividing cells, VC:VK<sub>3</sub> appears to target tumor cells by inflammation [23]. Inflammation is regarded as a "secret killer" and is present in the microenvironment of most neoplastic tissues [28]. A wide variety of stimuli including: microbial infections, viral infections and autoimmune disease can trigger chronic inflammation and the subsequent development of cancer [29]. Chemokine and cytokine production orchestrated by inflammation-sensitive transcription factors are the key players in this cancer-related inflammation (CRI) [30-32] and its role in tumor initiation, promotion, invasion, and metastasis [33]. Therefore, inflammation can be considered an enabling characteristic for the acquisition of the core properties of cancer

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**Received** August 19, 2013; **Accepted** October 07, 2013; **Published** October 09, 2013

**Citation:** McGuire K, Jamison J, Gilloteaux J, Summers JL (2013) Vitamin C and K<sub>3</sub> Combination Causes Enhanced Anticancer Activity against RT-4 Bladder Cancer Cells. J Cancer Sci Ther 5: 325-333. doi:[10.4172/1948-5956.1000223](http://dx.doi.org/10.4172/1948-5956.1000223)

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[28]. Furthermore, because smoldering inflammation in the tumor microenvironment influences responses to chemotherapy as well as hormonal therapy and is involved in the pathogenesis of many types of cancer including bladder cancer, CRI represents a target for therapeutic intervention [28,34]. For example, tumor cells possess a greater need for glucose than normal cells and express facilitative glucose transporters (GLUTs) to achieve this task. Because of the structural resemblance of dehydroascorbic acid (DHA, the oxidized form of vitamin C) to glucose, DHA can also enter the tumor cells through the GLUT transporters and bioaccumulate. Epithelial tumors appear to rely on superoxide (inflammation) which is produced constitutively via NADPH oxidase of non-neoplastic stromal cells to oxidize the ascorbic acid to DHA [35]. Once dehydroascorbic acid enters the cells, it is reduced and retained as ascorbic acid (AA) which is not transportable through the bidirectional GLUTs [36]. Subsequently, AA, DHA or their metabolites inhibit many cellular processes, including glycolysis [37]. Because of the Warburg effect during which cancer cell metabolism becomes more reliant on glycolysis than mitochondrial oxidative respiration, the VC:VK<sub>3</sub> combination is able to exploit both inflammation and tumor metabolism in a multi-pronged strategy against a variety of tumor cells, including RT4 cells, that results in a new type of cell death termed autophagocytosis [17,20,26,27]. These results have been extended into the clinical setting with a phase I/IIa clinical trial for end stage prostate cancer which demonstrated both safety and efficacy [38]. The results presented here represent an initial investigation into the mechanism(s) responsible for autophagocytic cell death in grade I bladder cancer (RT-4) cells following VC:VK<sub>3</sub> treatment.

## Materials and Methods

### Cell lines

Grade I (RT-4) and grade III/IV (T24) human bladder cancer cell lines were purchased from the American Type Culture Collection (Rockville, MD, U.S.A.) and were grown in Eagle's minimum essential medium (MEM) and McCoy's 5A respectively (Gibco, Grand Island, NY, U.S.A.). All media was supplemented with 10% fetal bovine serum (Gibco) and 50 µg/mL Gentamycin sulfate (Sigma Aldrich, St. Louis, MO).

### Test solutions

Sodium L-Ascorbate (VC) and menadione sodium bisulfite (VK<sub>3</sub>) were purchased from Sigma Chemical Company (St Louis, MO, U.S.A.) and were dissolved in 1X phosphate-buffered saline (PBS). For the cytotoxicity assay vitamins were diluted to a final concentration of 10,000 µM VC and 500 µM VK<sub>3</sub> alone and the combination was diluted to a final concentration of 8,000 µM VC and 80 µM VK<sub>3</sub> respectively. Two fold serially dilutions were then performed and solutions were added to the plate. The CD<sub>50</sub> concentrations determined by the cytotoxicity assay [VC (8,750 µM), VK<sub>3</sub> (90 µM) and VC:VK<sub>3</sub> (520 µM:5.2 µM)] were used for all additional experiments.

### Protein concentration assay

Total protein content for each sample was determined using the method of Bradford [39] and sham treated cells served as control for all experiments.

### Cytotoxicity assay

Tumor-cell cytotoxicity was performed using the microtetrazolium assay [MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-diphenyltetrazolium bromide] as described previously [16]. Briefly,

corning 96-well titer plates were seeded with tumor cells (5 x 10<sup>3</sup> per well) and allowed to grow and spread overnight. Cells were consequently incubated for 1-h or 5-days with VC or VK<sub>3</sub> alone or in combination. Formazan crystals were dissolved in DMSO and plates were read at 590nm and 620nm on a Biotek Synergy HT plate reader. The CD<sub>50</sub> was determined based on the line of best fit. The fractional inhibitory concentration index (FIC) was employed to evaluate synergism.

### Analysis of protein thiols

Thiol levels were determined according to Nagelkerke et al. [40]. In brief, RT4 cells were treated with CD<sub>50</sub> concentrations of the vitamins alone or in combination. Cells were washed with PBS, culture media and trypsinized every hour up to 6 hrs. Cells were subsequently pelleted for 5 min at 1000 rpm, washed twice with 6.5% TCA (trichloroacetic acid) and resuspended in 1 mL of 0.5 M Tris-HCl (pH 7.6). To detect thiols 50 µL of 10 mM methanolic Ellman's Reagent was added to each sample and incubated for 20 min at room temperature. The solution was then centrifuged for 5 min at 1000 rpm and the absorbance of the resulting supernatant was measured at 412 nm. Thiol content was determined based on a reduced glutathione (GSH) standard curve and was expressed as µM thiols per mg of protein.

### Analysis of ATP

RT4 cells were seeded at a density of 1.0 x 10<sup>6</sup> and allowed to grow and spread overnight at 37°C and 5% CO<sub>2</sub>. Culture medium was removed; the cells were treated with vitamins alone or in combination and ATP content were determined every hour for 5 hrs. Cells were then washed with 1xPBS covered with vitamin-free media and solubilized in somatic cell ATP releasing reagent (Sigma Chemical Co, St Louis, MO). Cellular ATP content was determined using an ATP bioluminescent assay kit (Sigma, St. Louis, MO) [41] and bioluminescence was then measured using a Beckman LS 9000 scintillation counter set for single photon counting. ATP content was then calculated based on an ATP standard curve and was expressed as nM ATP per mg of protein.

### Lipid peroxidation

Lipid peroxidation was evaluated using the thiobarbituric acid (TBA) method [42]. RT4 cells were treated and harvested as described in the thiol assay. After centrifugation, the cell pellets were resuspended in 6.0% TCA (trichloroacetic acid), mixed with 1 ml of 0.25 N HCl containing 0.375% TBA and 15% TCA heated in a water bath for 15 min at 95°C and then allowed to cool. Following centrifugation the supernatant was monitored fluorimetrically for malondialdehyde (MDA) production using an excitation wavelength of 532 nm and an emission wavelength of 555 nm. Data was expressed as nM MDA per mg of protein, calculated on the basis of an MDA standard curve generated using 1, 1, 3, 3-tetramethoxypropane.

### Calcium

Calcium was assayed according to the method of Scott et al. [43]. Briefly, 4x10<sup>6</sup> cells were suspended in 1mL of calcium and magnesium free HBSS containing CD<sub>50</sub> vitamin concentrations and incubated at 37°C for 15, 30, 45 and 60 min. Following incubation the cell suspension was treated with 100 µL of 390 µM arsenazo III (2,2'-[1,8-dihydroxy-3,6-disulpho-2,7-naphthalene-bis(azo)]-dibenzene arsonic acid). Followed by the addition of 100 µL of 130 µM FCCP (carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone) to the cell suspension and the mitochondrial calcium release was recorded until no further change in absorbance was observed at 675-685 nm using a HP8451A diode array spectrophotometer. Then 100 µL of 195 mM A23187 (a calcium

ionophore) was added and extra mitochondrial calcium release was recorded until no further change in absorbance was recorded at 675-685 nm. Calcium concentration was determined using a calcium standard curve. The linear range of the standard curve was used to express the calcium concentration as nM calcium/mg protein [44].

## Statistics

A three-way ANOVA was performed using BMDP statistical software. In the three-way ANOVA, the two-way interactions were tested at the 0.005 level of significance. All other effects were tested at the 0.0022 level of significance.

## Results

VC, VK<sub>3</sub> and the combination of VC:VK<sub>3</sub> in a ratio of 100:1 have been evaluated for their cytotoxicity against both the low grade (RT-4) and high grade (T-24) bladder cancer cell lines following continuous 5-day vitamin exposure or 1-h vitamin exposure followed by a 5-day incubation in media (Table 1). A continuous 5-day vitamin treatment of the RT-4 cells resulted in CD<sub>50</sub> values of 2,430 μM for VC, 12.8 μM for VK<sub>3</sub> and 110 μM:1.10 μM for the VC:VK<sub>3</sub> combination. These results represented a 22-fold decrease of the CD<sub>50</sub> of VC and a 12-fold decrease in the CD<sub>50</sub> of the VK<sub>3</sub>. The fractional inhibitory concentration index (FIC) was used to assess the synergism of the combination. For the RT-4 cells, the VC:VK<sub>3</sub> resulted in an FIC value of 0.136. Continuous 5-day vitamin treatment of the T24 cells produced a CD<sub>50</sub> value of 1,490 μM for VC, 13.1 μM for VK<sub>3</sub> and 212 μM: 2.13 μM for VC:VK<sub>3</sub>. These results correspond to a 41-fold decrease of the CD<sub>50</sub> of VC and a 6-fold decrease in the CD<sub>50</sub> of the VK<sub>3</sub> with an FIC value of 0.158 (Table 1).

Studies by Taper and co-workers [11] demonstrate that exposure to the combination for as little as 1h, results in significant anti-tumor activity. A similar experiment was performed to determine if this effect was repeatable against RT-4 and T24 cells following a 1hr vitamin exposure. A 1-h vitamin treatment of the RT-4 cells resulted in CD<sub>50</sub> values of 4,740 μM for VC, 60.7 μM for VK<sub>3</sub> and 267 μM:2.68 μM for VC:VK<sub>3</sub>. These values correspond to an 18- fold decrease in the CD<sub>50</sub> value of VC, a 22-fold decrease in the CD<sub>50</sub> value of VK<sub>3</sub> and produced an FIC value of 0.100. In the case of the T24 cells, the CD<sub>50</sub> values were 4,970 μM for VC, 73.2 μM for VK<sub>3</sub> and 120 μM: 1.21 μM for VC:VK<sub>3</sub>. These values correspond to a 41-fold decrease in the CD<sub>50</sub> of VC and a 59-fold decrease in the CD<sub>50</sub> of the VK<sub>3</sub> with a corresponding FIC for the vitamin combination of 0.093 (Table 1).

VC, VK<sub>3</sub> or the VC:VK<sub>3</sub> combination has been shown to generate hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and other reactive oxygen species (ROS) in tumor cells and these species may initiate peroxidation of lipid membranes [15,17]. Vitamin induced lipid peroxidation (Figure 1) was examined using the thiobarbituric acid method. The lipid peroxidation of sham-treated RT-4 cells displayed an average value of 3.17nM (MDA)/mg of protein. However, this is only a measure of the lipid

peroxidation that occurs during the heating of samples to 95 °C during the assay and can, therefore, be considered as a baseline for MDA production. VC treatment resulted in MDA peak at 4.27 nM/mg with an average value of 3.67 nM/mg. Lipid peroxidation of VK<sub>3</sub>-treated cells was 4.34 nM/mg at 1 h increased to near 5.84 nM/mg by hours 3 and 4 and decreased to 4.27 by hour 5. The average VK<sub>3</sub>-induced lipid peroxidation was 4.98 nM/mg. This spike in lipid peroxidation was attributed to a concomitant increase in ROS production due to redox cycling and a decrease in catalase activity [45]. VC:VK<sub>3</sub> lipid peroxidation peaked at 6.7 nM/mg with an average value of 5.58 nM/mg of protein. Overall, the VC:VK<sub>3</sub> treatment resulted in a statistically significant alteration in lipid peroxidation (p<< 0.0022) for all time points compared to control.

Administration of VK<sub>3</sub> or menadione has been shown to cause depletion of GSH and oxidation of protein sulphhydryl groups in cytoskeletal proteins [46,47]. Therefore, the effect of vitamin treatment on cellular thiols has been examined (Figure 2). The sham-treated RT4 cells presented with an average thiol content of 1.39 ± 0.42 μM thiol/mg of protein. All other cells were exposed to the vitamins for 1 h and then incubated in vitamin-free culture media for 5 h. VC treatment in the first hour depleted thiol levels to 0.92 ± 0.31 μM thiol/mg of protein which was not statistically significant compared to the control value at 1 h. Thiol levels remained constant during the second hour and dropped steeply to 0.47 ± 0.03 μM thiol/mg of protein during the third hour. These levels rebounded to 0.73 ± 0.12 μM thiol/mg of protein during the fourth hour and then returned to second and third hour levels of 0.45 ± 0.03 μM thiol/mg of protein during the final hour. The values for the remaining hours are statistically significant (p << 0.0022) compared to their corresponding control values.

VK<sub>3</sub> treatment decreased thiol levels to 0.62 ± 0.5 μM thiol/mg of protein during the first hour, where they remained constant for the next three hours. By five hours thiol levels lowered slightly to 0.54 ± 0.1 μM thiol/mg of protein. The VC:VK<sub>3</sub> combination produced a stepped decrease in thiol levels during the first and second hour from 0.63 ± 0.05 μM thiol/mg to 0.45 ± 0.03 μM thiol/mg of protein. Overall, VC:VK<sub>3</sub> treated cells induced a significant (p<0.0022) depletion of cellular thiols.

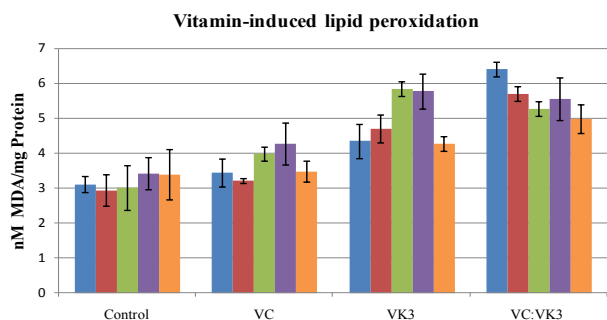
Investigation using transmission electron microscopy has shown that mitochondrial ultrastructure is altered by vitamin treatment resulting in autschizic cell death [15,48]. To determine the role of mitochondria in VC:VK<sub>3</sub> induced cell death the intracellular levels of ATP were measured over the course of 5hrs to look for an ATP-less cell death as a result of mitochondrial damage (Figure 3). The ATP content of sham treated RT-4 cells varies from 58.10 to 62.20 nM ATP/mg of protein with an average value of 59.64 ± 2.4 nM ATP/mg of protein. VC exposure results in an increase in ATP levels to 147 ± 8.64 nM during the first hour. Subsequently, the ATP levels decreased to 86.0

Cell Line	Incubation Time	Vitamins Alone		Vitamin Combination		FIC
		VC CD50 (μM)	VK <sub>3</sub> CD50 (μM)	VC CD50 (μM)	VK <sub>3</sub> CD50 (μM)	
RT4	1 h	4,740 ± 27.2	60.7 ± 4.01	267 ± 4.04	2.68 ± 0.05	0.100
	5 days	2,430 ± 28.3	12.8 ± 0.03	110 ± 9.73	1.10 ± 0.10	0.136
T24	1 h	4,970 ± 27.4	73.2 ± 5.91	120 ± 7.0	1.21 ± 0.07	0.093
	5 days	1,490 ± 141	13.1 ± 0.01	212 ± 7.6	2.13 ± 0.06	0.158

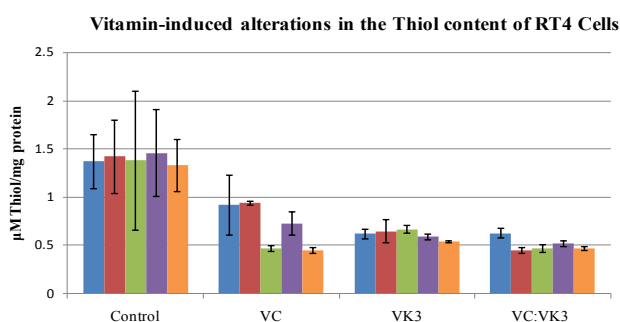
FIC = CD<sub>50</sub><sup>A comb</sup> / CD<sub>50</sub><sup>A alone</sup> + CD<sub>50</sub><sup>B comb</sup> / CD<sub>50</sub><sup>B alone</sup>, where CD<sub>50</sub><sup>A alone</sup> and CD<sub>50</sub><sup>B alone</sup> are 50% cytopathic doses of each vitamin alone; CD<sub>50</sub><sup>A comb</sup> and CD<sub>50</sub><sup>B comb</sup> are the 50% cytopathic doses of the vitamins administered together. FIC<1.0 is synergistic, FIC>1 is antagonistic and FIC=1 is indifferent

**Table 1:** Antitumor Activity of Vitamins Against RT-4 Bladder Carcinoma Cells.





**Figure 1:** RT-4 cells were treated with the vitamins at their CD<sub>90</sub> doses VC (8,750μM), VK<sub>3</sub> (90μM) and VC:VK<sub>3</sub> (520μM:5.2μM) and harvested at one hour intervals for 5 h and assayed for lipid peroxidation using the thiobarbituric acid method. Malondialdehyde (MDA) production was monitored fluorimetrically and data was expressed as nM MDA per mg of protein and calculated based on an MDA standard curve. Values are the mean ± standard error of the mean of three experiments with three readings per experiment and compared to the control. VC:VK<sub>3</sub> treatments resulted in significant amounts of lipid peroxidation compared to control ( $P < 0.0022$ ).



**Figure 2:** RT4 cells were treated for 1 hour with the vitamins at their CD<sub>90</sub> doses, VC (8,750μM), VK<sub>3</sub> (90μM) and VC:VK<sub>3</sub> (520μM:5.2μM) harvested at one hour intervals for 5 h and assayed for cellular thiol content by monitoring absorbance following reaction with Ellman's Reagent. Data has been expressed as μM Thiol/mg of protein, calculated on the basis of a GSH standard curve. Values are the mean ± standard error of the mean of three experiments with three readings per experiment. VC:VK<sub>3</sub> causes significant depletion of cellular thiols compared to the control ( $P < 0.0022$ ).

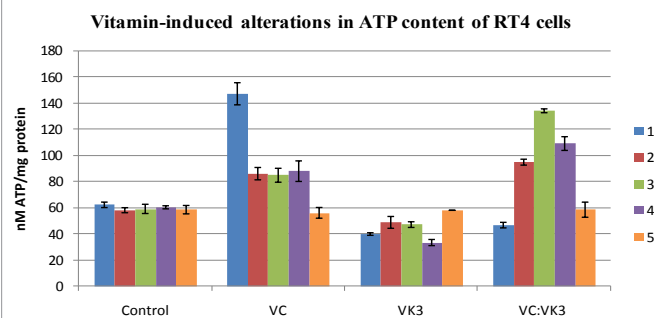
± 4.73 nM during the second hour and remained relatively constant during the third and fourth hours and then fell to 56.1 ± 4.09 nM during the final hour. VK<sub>3</sub> treatment lowered ATP levels to 39.9 ± 0.99 nM during the first hour. Subsequently, ATP levels rose slightly to 48.8 ± 4.52 nM during the second hour, remained relatively constant for the next 3 hours and increased to near control levels during the final hour.

The VC:VK<sub>3</sub> combination produced a slight decrease in ATP concentration to 46.7 ± 2.13 nM during the first hour. ATP levels increased during the second and third hours to 134 ± 1.46 nM and decreased gradually to near control levels during the final two hours. These results demonstrate that pulse treatment of RT-4 cells with VC alone or with the VC:VK<sub>3</sub> combination resulted in a transient increase in intracellular ATP levels following vitamin treatment. The treatment of the cells with the VC:VK<sub>3</sub> combination resulted in a significant ( $p < 0.0022$ ) alteration in ATP levels for all hours except for 5h when the difference from control is not significant.

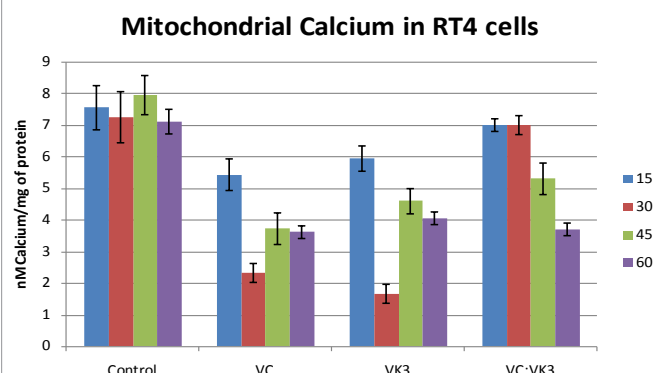
To determine the role of calcium in VC:VK<sub>3</sub> induced cell death the mitochondrial and extra mitochondrial calcium levels were measured

during the first 1hr to look for differences in apoptotic and autophagic calcium patterns (Figure 4 and 5). The mitochondrial calcium (Figure 4) content of sham treated RT-4 cells varies from 7.11 to 7.95 nM calcium/mg of protein with an average value of 7.47 ± 0.63 nM calcium/mg of protein. VC exposure results in a ~70% decrease in calcium levels to 2.32 ± 0.3 nM during the first thirty minutes compared to control. Subsequently, by 45min to 1 hour the calcium levels rebound to ~50% of control values with an average of 3.67 ± 0.35 nM. VK<sub>3</sub> treatment also lowered calcium levels by ~75% compared to control values to 1.66 ± 0.3 nM during the first thirty minutes. Subsequently, by 45min to 1 hour the calcium levels rebound to ~40% of control values with an average of 4.32 ± 0.3 nM by 1hr.

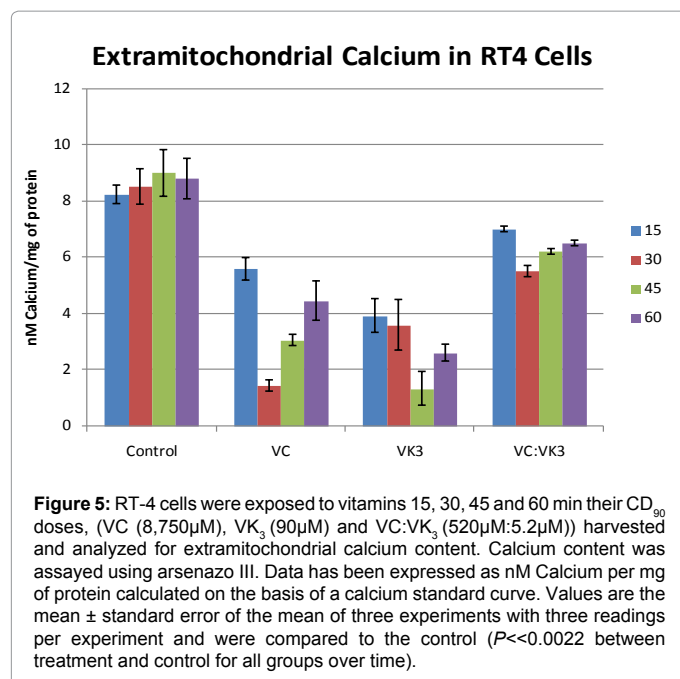
Unlike VC and VK<sub>3</sub> alone, the VC:VK<sub>3</sub> combination showed no statistically significant change in mitochondrial calcium concentration during the first thirty minutes with an average of 7.0 ± 0.25 nM. This lack of change in mitochondrial calcium concentration during the first 30 minutes probably reflects a slower rate of mitochondrial accumulation



**Figure 3:** RT-4 cells were treated for 1 hour with the vitamins at their CD<sub>90</sub> doses (VC (8,750μM), VK<sub>3</sub> (90μM) and VC:VK<sub>3</sub> (520μM:5.2μM)) and then harvested at one hour intervals for 5 h. ATP content was assayed using a bioluminescence assay. Data has been expressed as nM ATP per mg of protein and calculated on the basis of an ATP standard curve. Values are the mean ± standard error of the mean of three experiments with three readings per experiment and were compared to the control ( $P < 0.0022$  between VC:VK<sub>3</sub> and control from 2-4 hrs).



**Figure 4:** RT-4 cells were exposed to vitamins 15, 30, 45 and 60 min their CD<sub>90</sub> doses, VC (8,750μM), VK<sub>3</sub> (90μM) and VC:VK<sub>3</sub> (520μM:5.2μM) harvested and analyzed for mitochondrial calcium content. Calcium content was assayed using arsenazo III. Data has been expressed as nM Calcium per mg of protein calculated on the basis of a calcium standard curve. Values are the mean ± standard error of the mean of three experiments with three readings per experiment and were compared to the control ( $P < 0.0022$  between treatment and control for all groups by 45-60 minutes).



of the VC and VK<sub>3</sub> in the combination because their concentrations are 17-fold lower than that used in the vitamins alone. Subsequently, mitochondrial calcium levels slowly decrease to an average of  $4.5 \pm 0.35$  nM by 1hr never dropping below ~40% of control mitochondrial calcium levels. These results demonstrate that treatment of RT-4 cells with VC or VK<sub>3</sub> alone caused a transient release in mitochondrial calcium levels with levels subsequently rebounding to ~40-50% of the control value. The treatment of the cells with the VC:VK<sub>3</sub> combination displayed a calcium pattern that was distinctly different from the mitochondrial calcium pattern created by either vitamin administered alone with, combination mitochondrial calcium levels never dropping below 40% of control levels.

Changes in extra mitochondrial calcium were also examined to look for calcium release from other cellular compartments including the endoplasmic reticulum and cell membrane. The extra mitochondrial calcium content of sham treated RT-4 cells varies from 8.23 to 8.99 nM calcium/mg of protein with an average value of  $8.63 \pm 0.63$  nM calcium/mg of protein. VC exposure results in a ~80% decrease in calcium levels to  $1.43 \pm 0.2$  nM during the first thirty minutes compared to control. Subsequently, by 45min to 1 hour the calcium levels rebound to ~30-35% of control values with an average of  $3.75 \pm 0.45$  nM. VK<sub>3</sub> treatment also lowered calcium levels by ~55-60% compared to control values to an average of  $3.75 \pm 0.75$  nM during the first thirty minutes. Subsequently, by 45min the calcium levels plummet ~20% of control values with an average of  $1.33 \pm 0.6$  nM. Finally, by 1hr extra mitochondrial calcium levels slightly rebound to ~30% of control but remain low.

The VC:VK<sub>3</sub> combination showed a maximum ~36% decrease in extra mitochondrial calcium concentration by thirty minutes with an average of  $5.5 \pm 0.2$  nM. By 45 minutes to 1hr the extra mitochondrial calcium level slowly rises upward to reach ~75% of the control level. These results demonstrate that treatment of RT-4 cells with VC or VK<sub>3</sub> alone caused a decrease in extra mitochondrial calcium levels while the combination displayed only a slight decrease in extra mitochondrial calcium over the course of 1hr.

## Discussion

VC is cytotoxic to a variety of tumor types [49-51] when administered as a monotherapy or as a sensitizer of tumor cells to radiation and chemotherapy [49,52,53]. At megadoses, VC generates hydrogen peroxide, ROS, depletes cellular thiols and initiates lipid peroxidation (LPO). One problem commonly associated with VC therapy is achieving and maintaining clinically active doses in the bloodstream. For example, following oral VC administration, VC concentrations in the blood peak at ~220 μM which is below the concentration required for clinical efficacy. Conversely, following intravenous (iv) VC administration, VC concentrations in the blood peak at ~885 μM which is sufficient for clinical efficacy. However, the half-life of this iv dose of VC is short with circulating VC doses returning to control levels within 4 to 6 h [54]. This problem of achieving and maintaining clinically active doses in the bloodstream has hindered VC monotherapy from becoming a widely acceptable cancer therapy.

VK<sub>3</sub> also exhibits *in vitro* cytotoxic activity against a variety of tumor cell lines [55] as well as *in vivo* antitumor activity [56]. VK<sub>3</sub> can act to detoxify ROS (reduced environment) or act as a ROS generator (prooxidant environment) through single electron (1e<sup>-</sup>) and two electron (2e<sup>-</sup>) cycling. At doses greater than 50 μM, VK<sub>3</sub> causes tumor cell death [57] by depleting cellular pools of ADP, ATP and glutathione (GSH); inducing single stranded DNA breaks and oxidizing protein sulphydryl groups [50]. VK<sub>3</sub> is also a chemosensitizer for most traditional chemotherapeutic agents [58]. The MTD for menadione was determined in phase I and II studies to be 2.5 g/m<sup>2</sup>, but once the dose was increased to between 4 and 8 g/m<sup>2</sup> hemolysis occurred despite the presence of red blood cell glucose-6-phosphate dehydrogenase with no notable coagulopathy [59-61].

Combining VC and VK<sub>3</sub> in a ratio of 100:1 lowered the CD<sub>50</sub> values of VC and VK<sub>3</sub> 6 to 41 fold. This drop in CD<sub>50</sub> values places the effective concentration of both VC and VK<sub>3</sub> into a physiologically relevant range (Table 1). In addition, the combination is a more effective ROS generator than either of the constituents alone and targets tumor cells thus, avoiding indiscriminate redox damage. Finally, the VC:VK<sub>3</sub> combination is an effective chemotherapy and radiation sensitizer in hepatoma bearing mice [11-13]. Taper and his associates have shown that the VC:VK<sub>3</sub> combination exhibited antitumor activity with exposure times as short as 1 h [11]. The results of previous studies with bladder cancer and other tumor cell lines demonstrated that VC:VK<sub>3</sub> induced cell death via a caspase independent process that was not apoptosis [27,62,63]. In addition, VC:VK<sub>3</sub> treatment did not lead to the conversion of soluble LC3-I to autophagic vesicle associated LC3-II and thus tumor cell death was not due to autophagy [64-66]. Instead, cell death was due to autophagy with cathepsins, not caspases, as the cell executioners [65,66]. In the case of RT4 cells, the antitumor activity of VC:VK<sub>3</sub> was due to cell death by autophagy [20]. Ultrastructural studies of vitamin-treated RT4 cells undergoing autophagy revealed exaggerated membrane damage and an enucleation process in which the perikarya separated from the main cytoplasmic body by self-excision. These self-excisions continued until all that remained was an intact nucleus surrounded by a narrow rim of cytoplasm that contained damaged organelles. The nucleus exhibited nucleolar segregation and chromatin decondensation followed by nuclear karyolysis and karyolysis [20].

In previous studies, including those with RT4 cells, it was determined that H<sub>2</sub>O<sub>2</sub> and other ROS were essential effectors of VC, VK<sub>3</sub> and VC:VK<sub>3</sub> activity [46,67] and the anti-cancer activity of the

vitamins could be destroyed by addition of exogenous catalase at doses as low as 100 µg/mL [68]. While VC generated H<sub>2</sub>O<sub>2</sub> peroxide primarily outside of the cell and VK<sub>3</sub> generated primarily intracellular H<sub>2</sub>O<sub>2</sub>, the VC:VK<sub>3</sub> combination appeared to produce both extracellular and intracellular H<sub>2</sub>O<sub>2</sub> with total H<sub>2</sub>O<sub>2</sub> production being additive [45]. In addition, VC and VK<sub>3</sub> formed a redox pair resulting in both one and two electron cycling and the depletion of cellular thiols as well as the generation of hydrogen peroxide, superoxide and other ROS [23]. The fact that a greater amount of catalase was required to destroy the antitumor activity of VK<sub>3</sub> than was required to destroy the antitumor activity of the vitamin combination, suggested that while H<sub>2</sub>O<sub>2</sub> was involved in the mechanism of action of these vitamins, the enhanced antitumor activity of the vitamin combination was not simply due to an excessive increase in H<sub>2</sub>O<sub>2</sub> production.

In an initial attempt to elucidate the H<sub>2</sub>O<sub>2</sub>-mediated forces underlying these mechanism(s), tumor cells were treated with VC alone, VK<sub>3</sub> alone or with the VC:VK<sub>3</sub> combination for 1h to allow triggering of autophagocytosis. Subsequently, the vitamins were removed, culture medium was added and changes in thiol levels, lipid peroxidation and ATP content were monitored for 5hrs (a time by which most cells would be undergoing autophagocytosis). The effects of continuous vitamin exposure over a 5 h time period have already been described in a previous manuscript [68]. Since vitamin administration induces H<sub>2</sub>O<sub>2</sub> production, the amount of LPO has been evaluated. While the increase in lipid peroxidation values for cells were significantly higher than control levels after 1hr of vitamin exposure, significant levels of lipid peroxidation and damage to the cell membrane occur only after 2–3 hr vitamin exposure and suggest that wholesale, indiscriminate lipid peroxidation was a late event in the cell death process. However, TEM micrographs of RT4 cells, that were treated at the same time and dose as those employed in this paper, demonstrated that the architecture of the mitochondria, lysosomes and endoplasmic reticulum (ER) was rapidly altered by vitamin-induced lipid peroxidation and/or disruption of the glutathione redox balance in the ER as well as diminution of reduced thiols in the membranes of these organelles [20,45,66]. The resultant damage to the membranes as well as Ca<sup>2+</sup> transport channels of the lysosomes, mitochondria and ER membranes leads to increased intracellular Ca<sup>2+</sup> levels. Ca<sup>2+</sup> dysregulation also leads to the activation of a number of phospholipases, proteases, and DNases [69]. This Ca<sup>2+</sup> release occurs within the first 5 minutes of vitamin treatment and ultimately leads to cell death [68].

To further differentiate autophagocytosis from other types of cell death, vitamin-induced changes in ATP levels were determined. While ATP levels in sham treated cells remained constant, ATP levels in VK<sub>3</sub> treated cells show a steady decline (1.5 fold decrease) during the 5 h. VK<sub>3</sub> has been shown to induce either apoptosis or necrosis depending on the dose and duration of exposure [70]. At the dose employed in this study (90 µM) VK<sub>3</sub> would be expected to induce necrosis and the diminution of ATP levels is consistent with this expectation. Conversely, VC treatment and VC: VK<sub>3</sub> treatment led to spikes in ATP production. In the case of VC treatment, ATP levels increased rapidly in the first hour and then fell for the next 4hrs. For the VC:VK<sub>3</sub> combination, ATP levels increased 2.3 fold (compared to those of the control levels) and then fell to control levels over the next two hours which suggests that autophagocytosis is an active ATP-dependent process. VC accumulation in the majority of tumor cells is through GLUT transporters in the form of DHA which bio-accumulates and then is trapped when it is reduced back to AA which cannot be transported through the GLUT transporters. Likewise, VC accumulates in mitochondria via GLUT transporters in the same fashion it bio-

accumulated in the cytoplasm [71]. Once inside the mitochondria, VC can form a shunt around some of the defective regions of complex III of the electron transport chain and thus reconstitute a portion of ability to produce ATP that had been lost due to alterations in protein complexes in the electron transport chain during oncogenesis [72-74]. In the case of the vitamin combination, the doses of both vitamins are much lower than those employed with either vitamin alone, i.e. 17-fold for VC (8,750 µM / 520 µM) and 17-fold for VK<sub>3</sub> (90 µM/5.2 µM). Since VC is concentrating in the cytoplasm and inside the mitochondria via GLUT transporters, one would intuitively expect the higher dose VC to produce the threshold dose necessary to form the electron shunt before the lower dose VC. Thus, the 1h versus the 3h ATP peaks. In addition, Eleff and co-workers [74] have shown that, when VC and VK<sub>3</sub> in a ratio of 100:1 are administered, menadione accepts electrons from coenzyme Q (ubiquinone), shuttles them to ascorbate and then to cytochrome c. The shunt was able to bypass the antimycin-a-sensitive site in both forward and reversed electron transport; had two intact phosphorylation sites; and produced a shift from glycolytic activity to increased mitochondrial oxidative phosphorylation and a diminution of lactic acidosis. Therefore, the combination resulted in a production of more ATP than the shunt produced by VC alone. Thus, the bigger change in ATP production of the vitamin combination compared to VC alone. It is believed that the ATP generated by this process allows the cells “to commit suicide”.

## Conclusion

While VC traditionally is perceived as an antioxidant, it may also act as a pro-oxidant, increase DNA damage and induce cell death [75]. Vitamin K<sub>3</sub> is an oxidant that exhibits antitumor activity against a variety of tumor cell lines as well as human explants which are resistant to other types of chemotherapy [76]. When VC is combined with VK<sub>3</sub>, the interaction fosters redox cycling [77] which increases oxidative stress. Consequently, the antitumor activity of the vitamins in the combination is 12- to 24-fold greater than the individual vitamins for the RT-4 cells and 6- to 41-fold greater for the T24 cells. In previous studies with RT4 cells, administration of the vitamin combination was shown to induce the rapid production of H<sub>2</sub>O<sub>2</sub> [70] and ROS [45]. Hydrogen peroxide was implicated in the antitumor activity of the vitamin combination because addition of exogenous catalase (to neutralize the H<sub>2</sub>O<sub>2</sub>) was shown to abrogate their enhanced antitumor activity [68]. Within the first hour following combined vitamin treatment, this oxidative stress decreases cellular thiol levels to less than half those of sham-treated cells. Previous studies have shown that the resulting loss of protection against ROS is accompanied by the oxidation and subsequent disruption of cellular caspases (including caspase-3) as well as microtubules and other cytoskeletal proteins [18,19]. This cytoskeletal disorganization is reflected by blister and bleb formation as well as by acute distortions in tumor cell shape [20]. Because vitamin administration induces H<sub>2</sub>O<sub>2</sub> production, the amount of LPO has been evaluated. While the increase in lipid peroxidation values for cells were significantly higher than control levels after 1 hr of vitamin exposure, significant levels of lipid peroxidation and damage to the cell membrane occur only after 2–3 hr vitamin exposure and suggest that wholesale, indiscriminate lipid peroxidation was a late event in the cell death process. However, TEM micrographs demonstrated that the vitamin combination rapidly altered mitochondrial architecture and induced ultrastructural changes in both the smooth and rough endoplasmic reticulum (SER, RER) as well. As a consequence of the changes, Ca<sup>2+</sup> transport systems of the mitochondria, SER and RER are perturbed and there is an increase in intracellular Ca<sup>2+</sup> levels which



leads to the reactivation of DNases [78]. While lipid peroxidation and subsequent loss of membrane integrity may be responsible for the release of Ca<sup>2+</sup> into the cytoplasm, the fact that ATP production by the mitochondria increases 1h after VC treatment and 3h after combined vitamin treatment suggests that the Ca<sup>2+</sup> release occurs via modulation of the voltage-dependent anion channel (VDAC) [66]. In addition to these processes mentioned in this study, a number of cellular processes were affected by the presence of AA and especially DHA, including: modulation of signal transduction, cell cycle arrest and inhibition of glycolytic respiration, inhibition of metastasis [10,26,62,71,79,80]. Taken together these results indicate that autschizis (the type of cell death induced by the vitamin combination) entails the coordinated modulation of cell signaling and metabolism by VC, VK<sub>3</sub> in their various redox states coupled with the attack of H<sub>2</sub>O<sub>2</sub> and ROS on cellular thiols, membranes, cytoskeleton, and DNA that continues until cell death by self-morsellation ensues.

## Acknowledgements

This work was supported by a grant from The American Institute for Cancer Research and the Summa Foundation, Akron Ohio. We apologize to the many colleagues whose work was not cited due to space restrictions.

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**Citation:** McGuire K, Jamison J, Gilloteaux J, Summers JL (2013) Vitamin C and K<sub>3</sub> Combination Causes Enhanced Anticancer Activity against RT-4 Bladder Cancer Cells. J Cancer Sci Ther 5: 325-333. doi:[10.4172/1948-5956.1000223](https://doi.org/10.4172/1948-5956.1000223)

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